A Comparison of NO and N_2O Production by the Autotrophic Nitrifier *Nitrosomonas europaea* and the Heterotrophic Nitrifier *Alcaligenes faecalis*

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Soil microorganisms are important sources of the nitrogen trace gases NO and N_2O for the atmosphere. Present evidence suggests that autotrophic nitrifiers such as *Nitrosomonas europaea* are the primary producers of NO and N_2O in aerobic soils, whereas denitrifiers such as *Pseudomonas* spp. or *Alcaligenes* spp. are responsible for most of the NO and N_2O emissions from anaerobic soils. It has been shown that *Alcaligenes faecalis*, a bacterium common in both soil and water, is capable of concomitant heterotrophic nitrification and denitrification. This study was undertaken to determine whether heterotrophic nitrification might be as important a source of NO and N_2O as autotrophic nitrification. We compared the responses of *N. europaea* and *A. faecalis* to changes in partial O_2 pressure (pO_2) and to the presence of typical nitrification inhibitors. Maximal production of NO and N_2O occurred at low pO_2 values in cultures of both *N. europaea* (pO_2, 0.3 kPa) and *A. faecalis* (pO_2, 2 to 4 kPa). With *N. europaea* most of the NH_4^+ oxidized was converted to NO_2^-, with NO and N_2O accounting for 2.6 and 1% of the end product, respectively. With *A. faecalis* maximal production of NO occurred at a pO_2 of 2 kPa, and maximal production of N_2O occurred at a pO_2 of 4 kPa. At these low pO_2 values there was net nitrite consumption. Aerobically, *A. faecalis* produced approximately the same amount of NO but 10-fold more N_2O per cell than *N. europaea* did. Typical nitrification inhibitors were far less effective for reducing emissions of NO and N_2O by *A. faecalis* than for reducing emissions of NO and N_2O by *N. europaea*. *A. faecalis* produced much less NO and N_2O under denitrifying conditions than under nitrifying conditions, and the NO produced appeared to result primarily from chemical interactions involving NO_2^- at pH 6.95. Once much of the nitrite was consumed, the NO and N_2O produced were further reduced to N_2. Given the rates of NO and N_2O production reported here, our results suggest that heterotrophic nitrification may be a significant source of N_2O in aerobic to near-anaerobic soils and water.

Losses of nitrogen trace gases may result in decreasing fertility, especially in those ecosystems which have suffered disturbance (30). In addition, both nitric oxide (NO) and nitrous oxide (N_2O) affect the earth's atmosphere. In the troposphere NO controls the concentration of ozone, whereas N_2O behaves as a greenhouse gas. In the stratosphere N_2O causes destruction of ozone (18, 29). Efforts to estimate global or regional sources and sinks for NO and N_2O require an understanding of the mechanisms of the production of these compounds so that the variables controlling fluxes of these gases can be identified.

NO and N_2O are produced by a wide variety of organisms, including autotrophic nitrifiers, heterotrophic nitrifiers (including both bacteria and fungi), heterotrophic denitrifiers, dissimilatory nitrate reducers, nitrate respirers, plants, and algae. It has become clear that there is a good deal of overlap in the capabilities of the various categories of organisms. For example, current evidence suggests that many organisms, such as *Pseudomonas* spp. and *Alcaligenes faecalis*, which can denitrify under anaerobic conditions can also nitrify under aerobic conditions (5, 20, 24). The mechanisms of production of NO and N_2O by autotrophic nitrifiers such as *Nitrosomonas europaea* and heterotrophic denitrifiers such as *A. faecalis* are probably the same (i.e., reduction of NO_2^- [denitrification]) (22, 23). It is not altogether clear how strict

aerobic or anaerobic conditions must be to support these processes.

The results of most field studies have suggested that NO and N_2O fluxes from intact aerobic soils are primarily due to metabolism by autotrophic nitrifiers such as *N. europaea* (2, 28), although in acid forest soils heterotrophic nitrifiers may be responsible for these fluxes (19). In anaerobic soils NO and N_2O are produced by denitrifiers (1, 26). The net emission of either NO or N_2O from soils depends upon the relative importance of production and consumption processes and is, therefore, highly sensitive to physical variables, such as soil texture and water-filled pore space (9, 26). In most field studies it is not at all apparent whether autotrophic nitrifiers, denitrifiers, or perhaps denitrifiers behaving as heterotrophic nitrifiers are responsible for emissions of NO and N_2O. Probably all of these organisms play a role in producing N trace gases depending on subtle changes in soil physical and chemical conditions (6).

Heterotrophic nitrifiers have not generally been considered important sources of nitrogen trace gases in terrestrial or aquatic ecosystems, yet these organisms are widespread and may dominate nitrogen-cycling processes in some ecosystems (for example, acid forest soils) (25). *A. faecalis* is capable of producing NO, N_2O, and NO_2^- in medium containing both NH_4^+ and NO_3^- at partial O_2 pressures (pO_2 values) ranging from 20 kPa to almost anaerobic conditions, a process that has been referred to as aerobic denitrification (1). More recently, however, this production of NO and N_2O as well as NO_2^- has been shown to result from oxidation of

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NH_4^+ or organic N compounds, in which organic C is used as a carbon and energy source (5, 20). In this study we compared the abilities of the heterotrophic nitrifier *A. faecalis* and the autotrophic nitrifier *N. europaea* to produce N trace gases under various pO_2 conditions and in the presence of a variety of nitrification inhibitors.

MATERIALS AND METHODS

Culture conditions. *N. europaea* ATCC 19718 and *A. faecalis* ATCC 8750 were purchased from the American Type Culture Collection, Rockville, Md. *N. europaea* was maintained in a medium containing 0.5 g of $(NH_4)_2SO_4$, 1 g of K_2HPO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.3 g of NaCl, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 7.5 g of $CaCO_3$ in 1 liter of distilled water. *N. europaea* was grown in a medium containing 1 g of $(NH_4)_2SO_4$, 0.5 g of Na_2CO_3, 0.04 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of KH_2PO_4, 0.0005 g of ferric citrate, 0.1 ml of 0.05% phenol red solution (Flow Laboratories), and 11.9 g of HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma Chemical Co., St. Louis, Mo.) in 1 liter of distilled water adjusted to pH 8.15.

A. faecalis was grown in a citrate minimal medium containing 9.28 g of K_2HPO_4 and 1.81 g of KH_2PO_4 in 1 liter of distilled water adjusted to pH 6.95 and autoclaved prior to addition of the following filter-sterilized (pore size, 0.2 μm) reagents: 661 mg of $(NH_4)_2SO_4$, 24.2 mg of $Na_2MoO_4 \cdot 2H_2O$, 5.6 mg of $FeSO_4 \cdot 7H_2O$, 0.99 mg of $MnCl_2 \cdot 4H_2O$, 51.5 mg of $CaCl_2 \cdot 2H_2O$, 200 mg of $MgSO_4 \cdot 7H_2O$, and 1.18 g of sodium citrate. For denitrification studies performed with *A. faecalis*, KNO_2 was added to a final concentration of 0.5 to 1 mM.

Batch culture experiments. Batch culture experiments with continuous sparging were started by inoculating media (600 ml) with filtered and washed (unless otherwise stated), exponentially growing bacteria. Exponentially growing *N. europaea* cultures were produced by diluting aliquots of a maintenance culture 10-fold into growth medium and incubating the resulting preparations at 32°C for 2 to 3 days with shaking at 150 rpm until a cell density of 5×10^7 to 10×10^7 cells per ml was reached. Exponentially growing *A. faecalis* preparations were produced by growing cultures in citrate minimal medium inoculated from a Trypticase soy agar slant for approximately 2 days with shaking (150 rpm) at 32°C. Experimental cultures were incubated at 32°C and continuously sparged with mixtures of filtered (pore size, 0.2 μm) air and N_2 at a flow rate of 150 ml min^{-1}, with the gas flow rates and mixing ratios controlled by mass flow controllers (Tylan Corp., Torrance, Calif.).

Dead-cell controls were produced either by holding samples of exponentially growing cultures in a boiling water bath for 30 min or by sterilizing them in a microwave oven at 700 W for 5 min (14).

Batch culture experiments in serum bottles were started by diluting exponentially growing cultures, prepared as described above, eightfold into fresh media (20 ml) in 125-ml serum bottles. The bottles were sparged with filtered air-N_2 mixtures for 30 min after the bottles were capped.

Batch culture experiments in flasks without sparging were prepared by diluting filtered and washed, exponentially growing cultures, prepared as described above, into fresh media (100 ml) in 500-ml flasks. When anaerobic conditions were required, the medium was kept in a Coy anaerobic chamber for 2 days prior to the start of the experiment. The filtered cells and all amendments were added in the anaerobic chamber before the flasks were sealed.

Determination of pO_2 in medium. A peristaltic pump was used to pump medium that was continuously sparged with air-N_2 mixtures past a Clark type dissolved oxygen (DO) microelectrode (Microelectrodes, Inc., Londonderry, N.H.) whose Teflon membrane was cemented into a 0.125-in. (0.32-cm) polyethylene tee. Prior to the start of the experiment, the tee was sterilized with ethanol (70%, vol/vol) and rinsed with sterile, distilled water. Calibration was performed before bacteria were added by bubbling the medium with N_2 for at least 2 h and setting the DO meter on zero. The DO meter was then set at full scale after the medium was sparged with either 1 kPa of O_2 in N_2 or breathing air. The sparging was continued until the reading stabilized. At the end of the experiment the DO electrode was again placed in sterile medium sparged with N_2 in order to determine whether the zero point of the DO meter had drifted. Over an 8-h period we observed drift of approximately 10% of full scale when 1 kPa of O_2 was used to set full scale and drift of 1 to 2% when 20 kPa of O_2 was used to set full scale.

Bacterial enumeration. *N. europaea* cultures were counted directly either in a Petroff-Hausser chamber by using phase-contrast microscopy and a magnification of ×540 or on filters (pore size, 0.2 μm; Nuclepore) by using an epifluorescence microscope after staining with acridine orange. For *A. faecalis* enumerations, a standard curve was constructed for optical density at 660 nm versus cell number, as determined by serial dilution and plating on Trypticase soy agar; cell counts were based on optical densities at 660 nm.

Chemical analyses. Ammonia was determined by the method of Solorzano (27). Nitrite and nitrate were analyzed as described by Parsons et al. (21). Samples taken for these determinations were filtered (pore size, 0.2 μm) and stored refrigerated in sterile vials until analyses were performed.

Samples for N_2O analysis were taken with disposable plastic syringes equipped with rubber plunger tips and three-way delrin stopcocks. Samples (10 ml) were injected within 30 min into a 1-ml gas sampling loop of a gas chromatograph (model 5890; Hewlett Packard) equipped with an electron capture detector and a packed Poropak Q column (3.5 m by 0.3 cm). The detector temperature was 330°C and the oven temperature was 50°C. Argon containing methane (5%) was supplied as the carrier gas at a flow rate of 30 ml min^{-1}.

Samples for analysis of NO were taken with disposable plastic syringes equipped with rubber-tipped plungers and three-way delrin stopcocks. These samples were analyzed immediately. A nitrogen dioxide detector (model LMA-3; Scintrex/Unisearch, Toronto, Canada) was modified to analyze the NO in syringe samples as shown in Fig. 1. The internal pump of the model LMA-3 detector was disabled to allow control of the air flow into the instrument with mass flow controllers (model FC-280; Tylan). The airstream (1,097 ml min^{-1}) was directed by a three-way stainless steel valve either through a CrO_3 converter which converts NO to NO_2 with 100% efficiency provided that the humidity of the airstream is approximately 25% (3) or through a blank tube. The converter tube was made of opaque tubing (7.6 cm by 0.64 cm [outside diameter]) packed with 10% chromium trioxide on 30/60-mesh firebrick (Chromosorb P; Johns Manville Corp.), prepared as described by Levaggi et al. (16). The airstream was passed through a Nafion drier (Permapure, Inc., Toms River, N.J.) packed with indicating silica gel to control humidity. Calibration standards were freshly prepared before each experiment by mixing an NO standard (Scott Environmental Technology, Plumsteadville, Pa.) with breathing air (Air Products, Hampton, Va.) by
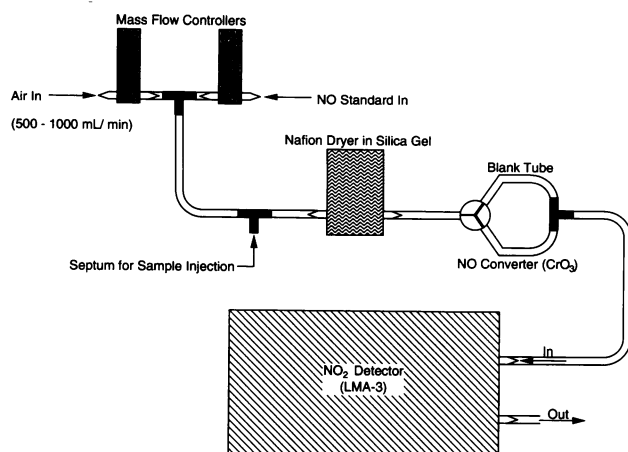
FIG. 1. Nitric oxide detection system. This system utilizes a nitrogen dioxide detector designed for continuous field monitoring. It was modified for analysis of nitric oxide in syringe samples taken during laboratory experiments. Nitric oxide in samples is converted to NO_2 by oxidation in a column containing CrO_3 on firebrick. The concentration of NO is equal to the difference between what is measured in the converted stream and what is measured in the unconverted stream.

FIG. 2. NO production and N_2O production by *N. europaea* as a function of pO_2 in the sparging gas. An exponentially growing culture of *N. europaea* was diluted 10-fold with growth medium and incubated at 32°C. The medium was continuously sparged at a flow rate of 150 ml min^{-1} with various mixtures of N_2 and breathing air. Following a change in the gas mixture, the culture was allowed to equilibrate for at least 30 min. pO_2 was measured continuously by pumping medium past a Clark type oxygen microelectrode. Syringe samples were taken from the headspace for analysis of NO and N_2O. Symbols: ●, NO; ▲, N_2O.

using mass flow controllers (Tylan). Standards were prepared daily and were kept in 1-liter Tedlar bags fitted with both a sampling septum and a gas inlet (Scott Environmental Technology). Volumes of gas standards (1 to 5 ml) were injected through a silicone septum into the airstream. When NO standards were stored in Tedlar bags over a 3-day period, there was approximately a 10% loss of NO in the highest concentration standard used (963 parts per billion, by volume).

The level of precision for replicate NO samples was typically 0.8%. The detection limit was 0.02 ng of N. When standards or samples containing high concentrations of NO (>3 ng of N) were injected into the instrument, we found that the luminol solution purchased from Scintrex/Unisearch became saturated, and the sensitivity of the instrument declined. A solution containing a 10-fold-higher concentration of luminol (10^{-3} M) in methanol was prepared as described by Burkhardt et al. (4) and was modified as suggested by Don Stedman (27a) for use in analyzing samples containing high concentrations of NO.

RESULTS

Previous studies on the effects of pO_2 on production of NO and N_2O by autotrophic nitrifiers have produced contradictory results (1, 17, 23). In order to resolve these differences, we utilized an experimental design in which culture medium was continuously recirculated past an oxygen microelectrode. This approach permitted more accurate determinations of pO_2 values in our experiments than were possible in previous studies. When early-stationary-phase cultures of *N. europaea* were diluted 10-fold into fresh medium and continuously sparged with air-N_2 mixtures having various pO_2 values, they exhibited a pO_2 optimum of 0.2 to 0.4 kPa for production of both NO and N_2O (Fig. 2). After dilution these cultures contained low concentrations of nitrite (0.2 to 0.5 mM). In contrast, when filtered and washed cells were used to inoculate similar cultures, production of NO and N_2O was undetectable, suggesting that the presence of
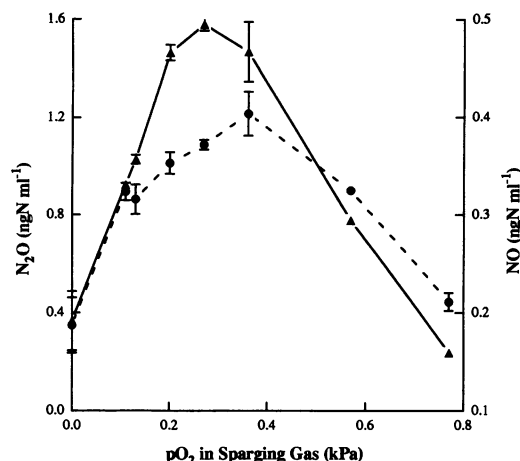
nitrite was necessary for production of both NO and N_2O. To determine the relative influence of pO_2 and nitrite concentrations on production of NO and N_2O, filtered and washed, exponentially growing *N. europaea* cells were added to serum vials containing either 1 or 20 mM nitrite. For each concentration of nitrite, one-half of the vials were sparged with 0.5 kPa of O_2, and the other half were sparged with 5 kPa of O_2. As shown in Fig. 3, production of both NO and N_2O varied as a function of NO_2^- concentration rather than as a function of pO_2. Dead-cell controls produced negligible amounts of both NO and N_2O.

In order to further examine the role of NO_2^- in the production of NO and N_2O by *N. europaea*, we measured production of these gases under anaerobic conditions by using various combinations of natural and artificial electron donors and acceptors. A combination of trimethylhydroquinone (TMHQ) (1 mM) as the electron donor and NO_2^- (1 mM) as the electron acceptor under anaerobic conditions was used to test whether NO_2^- could serve as an electron acceptor and source of NO or N_2O N during nitrification. The concentrations of NO and N_2O produced when TMHQ was the electron donor were somewhat less than the concentrations produced when we used hydroxylamine (NH_2OH) (1 mM), the natural electron donor, during nitrification (Fig. 4). Cell-free controls produced negligible amounts of NO and N_2O in the presence of TMHQ and NO_2^-.

In an attempt to determine whether NO or N_2O N is produced during oxidation of NH_4^+ or NH_2OH rather than during reduction of NO_2^-, we transferred filtered and washed, exponentially growing *N. europaea* cells to anaerobic medium containing either NH_4^+ (1 mM) or NH_2OH (1 mM) as the electron donor and phenazinemethosulfate (PMS) (100 μM) as the electron acceptor. As expected, neither NO nor N_2O was produced when NH_4^+ was the electron donor since NH_4^+ oxidation is obligately aerobic.
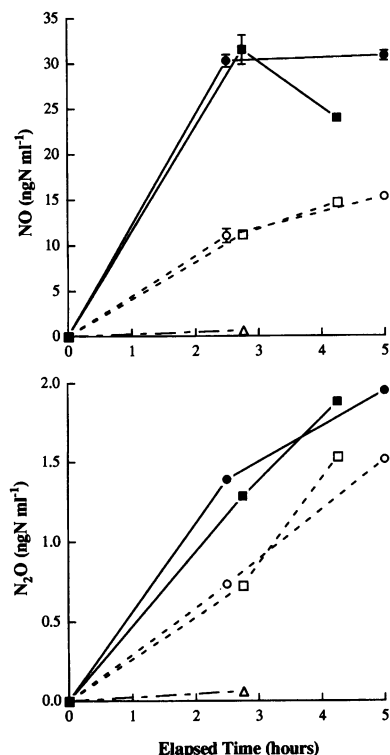
FIG. 3. NO production and N_2O production as a function of NO_2^- concentration and pO_2. An exponentially growing culture of *N. europaea* was filtered, washed, and diluted eightfold with growth medium containing NH_4^+ (15.2 mM) and NO_2^- (either 1 or 20 mM). Cultures were sealed in serum vials and sparged with either 0.5 or 5 kPa of O_2 for 30 min. The control vial had no cells in a medium containing 20 mM NO_2^- and a pO_2 of 5 kPa. Symbols: ○, 0.5% O_2, 1 mM NO_2^-; ●, 0.5% O_2, 20 mM NO_2^-; □, 5% O_2, 1 mM NO_2^-; ■, 5% O_2, 20 mM NO_2^-; △, control.

FIG. 4. Anaerobic production of NO and N_2O by *N. europaea* in the presence of the artificial electron donor TMHQ and the natural electron donor hydroxylamine. In both cases the electron acceptor was nitrite. Exponentially growing cells were filtered, washed, and diluted 10-fold with growth medium containing NO_2^- (1 mM) and either TMHQ (1 mM) or NH_2OH (1 mM). The flasks were degassed and kept in a Coy anaerobic chamber for the duration of the experiment. Control flasks contained no cells. Symbols: ○, TMHQ plus NO_2^-; ●, NH_2OH plus NO_2^-; △, TMHQ plus NO_2^- control; ▲, NH_2OH plus NO_2^- control.

However, when NH_2OH (1 mM) was the electron donor and PMS was the electron acceptor, NO was produced at a rate roughly equal to the rate observed when NO_2^- was used as the electron acceptor, and N_2O was emitted at a much higher rate in the presence of PMS than in the presence of NO_2^- (Fig. 5). Unfortunately, these results are equivocal since the product of NH_2OH oxidation is NO_2^-, which was available for reduction to NO and N_2O.

Although nitrification inhibitors such as N-serve and allylthiourea have been used in agriculture and are often used in field studies, the effectiveness of these compounds has been questioned (10). The intent of the experiments described below was to test the effectiveness of these inhibitors on production of NO and N_2O from NO_2^- by *N. europaea* under reduced oxygen conditions. In the presence of the nitrification inhibitors N-serve (10 μg ml^{-1} in 0.095% ethanol), allylthiourea (100 μM), and phenylacetylene (5 μg ml^{-1} in 0.095% ethanol), production of NO (Fig. 6) was inhibited, and there was no net increase in NO_2^- (data not shown) in cultures of *N. europaea* exponentially growing in medium containing 0.5 mM NO_2^- under a headspace containing 1 kPa of O_2. Small amounts of N_2O, which may have been produced abiotically, were emitted in the presence of these inhibitors (Fig. 6).

In order to compare the capabilities of heterotrophic and autotrophic nitrifiers to produce N trace gases, the heterotrophic nitrifier *A. faecalis* was exposed to many of the same
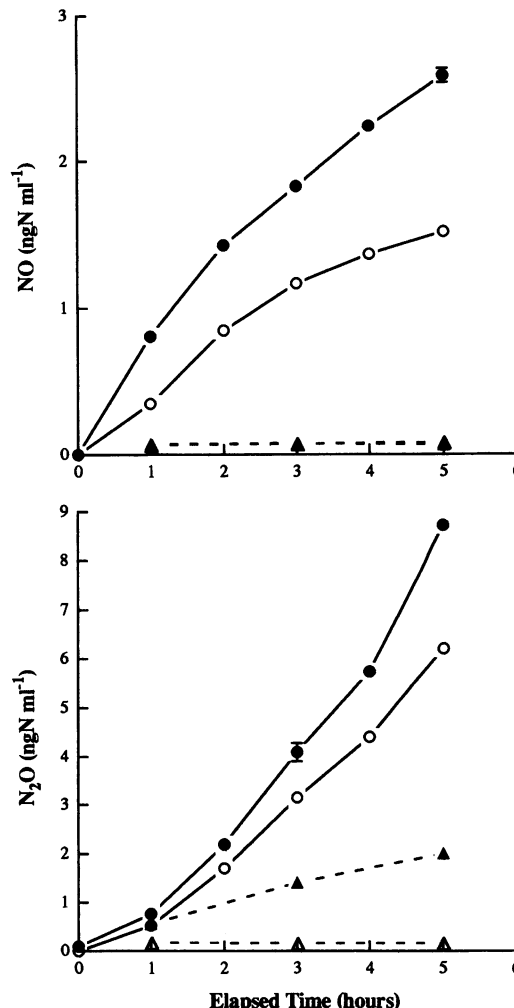
variables described above for *N. europaea*. When early-stationary-phase *A. faecalis* cells were filtered, washed, resuspended in fresh medium containing citrate (4 mM) as a carbon source and NH_4^+ as the sole nitrogen source, and shaken at 150 rpm, the NH_4^+ was oxidized to NO, N_2O, and NO_2^- (Fig. 7). As the cells entered the stationary phase (data not shown), both NO_2^- and NO were consumed, whereas N_2O continued to be produced at a linear rate (Fig. 7). The ratio of amount of NO produced to amount of N_2O produced during the linear phase of growth was 0.14 (standard deviation, 0.04). This contrasts with observations made during autotrophic nitrification, where the ratio of amount of NO produced to amount of N_2O produced was usually greater than 1. Under aerobic conditions *A. faecalis* was not able to utilize NO_2^- as a nitrogen source and therefore was not able to grow in medium containing citrate (4 mM) and
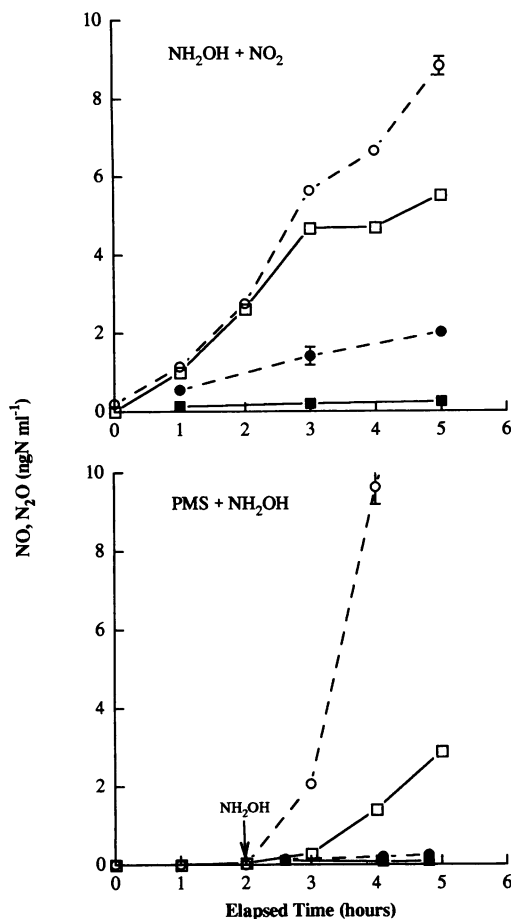
FIG. 5. Anaerobic production of NO and N_2O by *N. europaea* in the presence of the natural electron donor NH_2OH and either the natural electron acceptor NO_2^- or the artificial electron acceptor PMS. Exponentially growing cells were filtered, washed, and diluted 25-fold with growth medium containing NH_2OH (1 mM) and either NO_2^- (1 mM) or PMS (100 μM). The flasks were degassed and kept in an anaerobic Coy chamber for the duration of the experiment. Control flasks contained no cells. Symbols: O, N_2O; □, NO; ●, N_2O control; ■, NO control.

FIG. 6. Production of NO and N_2O by *N. europaea* in the presence of nitrification inhibitors under aerobic conditions. Exponentially growing cells were filtered, washed, and diluted 10-fold with growth medium containing NH_4^+ (15.2 mM), $NaCO_3$ (0.01%), NO_2^- (0.5 mM), and either N-serve (10 μg ml^{-1} in 0.095% ethanol), allylthiourea (100 μM), or phenylacetylene (5 μg ml^{-1} in 0.095% ethanol). The flasks were sealed, sparged with 1 kPa of O_2 in N_2, and incubated at 30°C with shaking (150 rpm). Symbols: O, no inhibitor; ●, N-serve; ∇, allylthiourea; ▼, phenylacetylene.

NO_2^- (0.5 mM) but lacking NH_4^+. Under these conditions no NO or N_2O was emitted (data not shown).

When an exponentially growing *A. faecalis* culture was continuously sparged with various mixtures of air and N_2, the optimum pO_2 for production of both NO and N_2O ranged from 2 to 4 kPa (Fig. 8). Over the range of pO_2 values studied (1 to 15 kPa), the ratio of amount of NO produced to amount of N_2O produced varied from 0.17 to 1.1, although we expected that this ratio would increase if we used higher flow rates for the sparging gas (31).

Under anaerobic conditions *A. faecalis* produced N_2O by denitrification of NO_2^- (Fig. 9) but not by denitrification of NO_3^- (data not shown). During denitrification N_2O was both produced and consumed. Dead cells did not consume NO_2^-, nor did they produce any N_2O. On the other hand, NO production was as high in the dead-cell control as it was in the experimental culture in the presence of 1 mM NO_2^-, suggesting that anaerobic production of NO resulted from chemodenitrification; however, the experimental culture consumed NO, whereas the dead-cell control did not (Fig. 9).
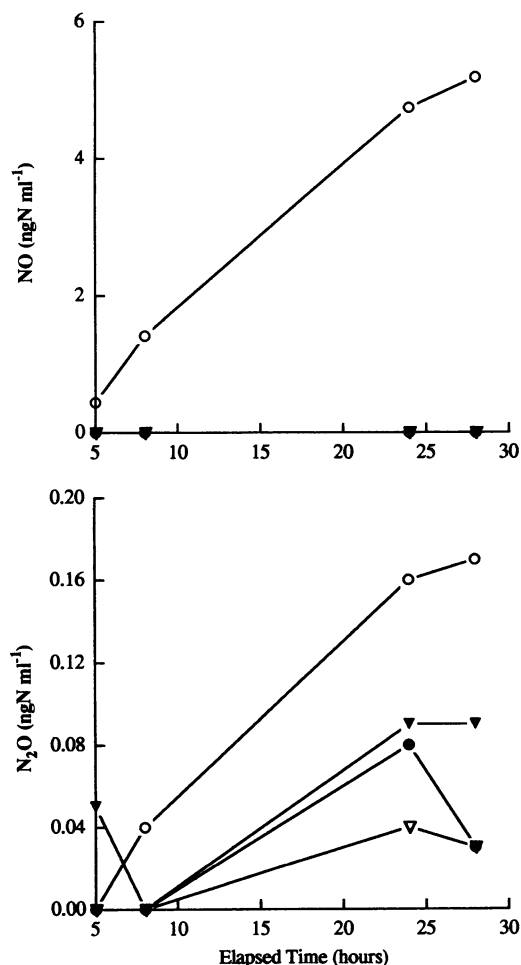
During heterotrophic nitrification by *A. faecalis*, N-serve (10 μg ml^{-1} in 0.095% ethanol), allylthiourea (100 μM), and phenylacetylene (5 μg ml^{-1} in 0.095% ethanol) had no effect on NO production or growth (as measured by optical density at 660 nm). However, N_2O production appeared to be inhibited, especially by N-serve and phenylacetylene; N_2O production was at least partially inhibited by allylthiourea (Fig. 10). N-serve and ethanol alone prevented accumulation of nitrite; however, nitrite production was only partially inhibited in the presence of phenylacetylene and allylthiourea (data not shown).

A summary and comparison of the results of the experiments described above (Table 1) showed that in cultures of *N. europaea* continuously sparged with 5 kPa of O_2 and in cultures of *A. faecalis* continuously sparged at a pO_2 of 1.6 kPa, net levels of production of NO, normalized to cell number, were approximately equal; however, net emission of N_2O by the heterotrophic nitrifier was approximately 10-fold greater than net emission of N_2O by the autotrophic
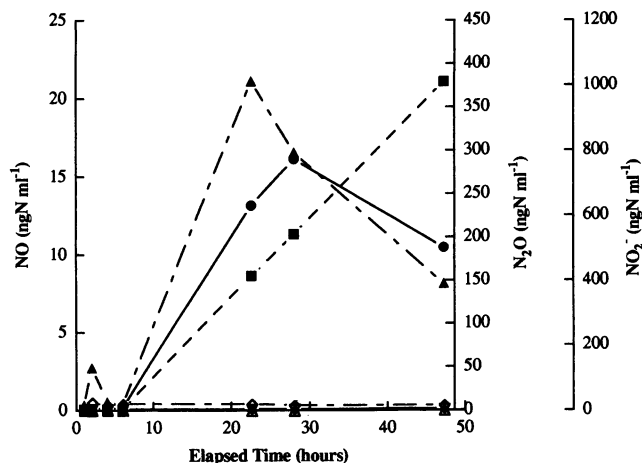
FIG. 7. Production of NO, N_2O, and NO_2^- by *A. faecalis* under aerobic conditions. Early-stationary-phase cells were filtered, washed, and diluted 100-fold with denitrification medium containing citrate (4 mM) and NH_4^+ (5 mM) but lacking NO_2^- and NO_3^-. Cultures were incubated at 36.5°C with shaking (150 rpm). Symbols: ●, NO; ■, N_2O; ▲, NO_2^-; ▽, NO, dead cells; △, N_2O, dead cells; ◇, NO_2^-, dead cells.

nitrifier. It could not be determined from these experiments whether the differences in net emissions were due to variations in the production rates or the consumption rates for these gases. When nitrite concentrations were normalized to cell number in the *A. faecalis* cultures, they showed net consumption of NO_2^- during incubation both in aerobic cultures and in anaerobic cultures. In the *N. europaea* cultures there was net accumulation of nitrite throughout the course of the experiment.

DISCUSSION

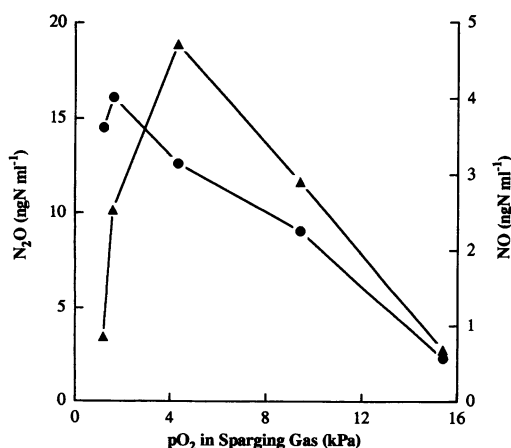Estimates of nitrogen trace gas emission have been made for a variety of temperate and tropical ecosystems, ranging

FIG. 8. Production of NO and N_2O by *A. faecalis* as a function of the pO_2 of the sparging gas. An early-stationary-phase culture was filtered, washed, and diluted 300-fold with medium containing citrate (4 mM) and NH_4^+ (5 mM). The culture was continuously sparged with air-N_2 mixtures at a flow rate of 150 ml min^{-1} and incubated at 31°C with shaking (125 rpm). The pO_2 was continuously monitored by pumping medium past a Clark type oxygen microelectrode. Symbols: ▲, N_2O; ●, NO.
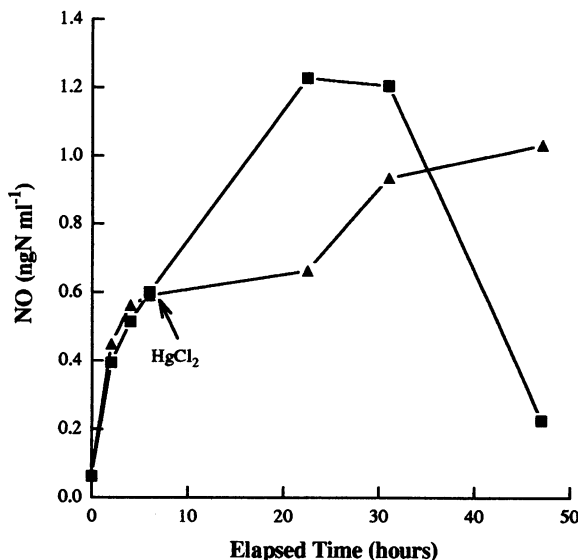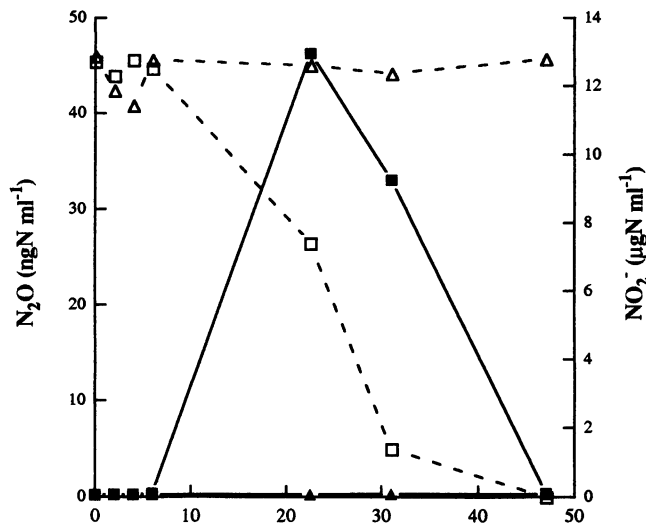

FIG. 9. Production of NO and N_2O and disappearance of NO_2^- in cultures of *A. faecalis* incubated anaerobically. An early-stationary-phase culture was filtered, washed, and diluted 28-fold with anaerobic medium containing citrate (4 mM), NH_4^+ (5 mM), and NO_2^- (1 mM). Manipulations were performed in an anaerobic Coy chamber. Cultures were incubated at 36.8°C with shaking (150 rpm). After 6 h of incubation, 1 ml of saturated $HgCl_2$ was added to the dead-cell control. Cells for the control were killed by boiling them for 30 min. Symbols: ▲, N_2O or NO, dead cells; ■, N_2O or NO, live cells; □, NO_2^-, live cells; △, NO_2^-, dead cells.

from seasonally dry forest to rain forest, yet it has been difficult to identify the critical environmental parameters which regulate fluxes of NO and N_2O over broad ranges of conditions (6, 9). Firestone and Davidson (7) have proposed a hole-in-the-pipe model suggesting that NO and N_2O fluxes are controlled at three levels; level one determines the relative rates of nitrification and denitrification, level two determines the relative proportions of NO and N_2O emitted from soils during either nitrification or denitrification, and level three determines the diffusion of these gases from the site of production to the atmosphere. The availability of O_2 to microorganisms is likely to be the critical parameter
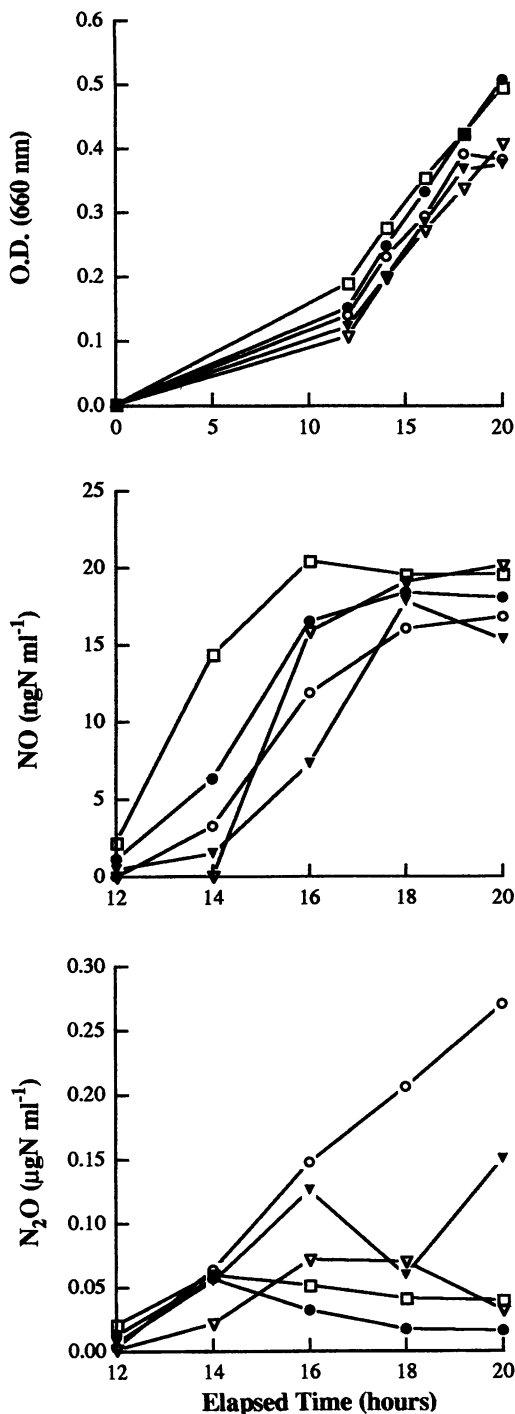
TABLE 1. Production of NO, N_2O, and NO_2^- by autotrophic and heterotrophic nitrifier-denitrifiers

Organism	Net production (ng of N day⁻¹ [10⁶ cells]⁻¹)			% of NO_2^- produced	
	NO	N_2O	NO_2^-	NO	N_2O
N. europaea[a]	21	8	809	2.6	1.0
A. faecalis[b]	17	95	—[c]		
A. faecalis[d]	0.1[e]	6.7	—[f]		

[a] N. europaea cultures were continuously sparged with 5% O_2 in N_2 at a flow rate of 150 ml min⁻¹.

[b] A. faecalis cultures at a pO_2 of 1.6 kPa were continuously sparged at a flow rate of 150 ml min⁻¹.

[c] NO_2^- production per 10⁶ cells declined over the 24-h period of the experiment.

[d] A. faecalis cultures were grown unsparged in a flask containing N_2.

[e] NO production was by chemodenitrification.

[f] NO_2^- was completely consumed over the 24-h period of the experiment.

affecting both level one and level two. A number of investigators have observed that pO_2 determines both the relative rates of nitrification and denitrification and also the ratio of amount of NO produced to amount of N_2O produced (1, 8, 13, 17, 23). The results of these experiments have often been contradictory; for example, Remde and Conrad (23) and Anderson and Levine (1) observed that NO emissions from cultures of nitrifiers are independent of pO_2, whereas Lipschultz et al. (17) noted an inverse relationship between NO emissions and pO_2. Our results suggest a solution to these contradictions. In sparged cultures the pO_2 optimum of 0.2 to 0.4 kPa for both NO and N_2O production observed in our experiments disappeared when NO_2^- concentrations greater than 0.5 mM were added to the growth medium, suggesting that NO_2^- and O_2 can compete for electrons removed during nitrification. Using partially purified extracts of nitrite reductase from N. europaea, Hooper (11) observed NO_2^- inhibition of O_2 utilization during NH_2OH oxidation.

Poth and Focht (22) were the first workers to demonstrate that NO_2^- can serve as an electron acceptor during nitrification with resultant production of N_2O. Our results demonstrate that NO, like N_2O, is produced during reduction of NO_2^- when TMHQ is used as an electron donor under anaerobic conditions. Using hydrazine as an electron donor, Remde and Conrad (23) similarly showed that during nitrification most of the NO produced is produced by nitrifier denitrification.

Under most field conditions, concentrations of nitrite in the soil are very low; thus, most investigators disregard nitrite as a possible controller of N trace gas emissions. When soils in arid ecosystems are first wetted, either artificially or by the first rains of the wet season, there are large but transient fluxes of NO (3). It has been suggested by Davidson (6) that nitrite, concentrated in thin water films around sites of nitrification during drying, might react abiologically with organic matter to produce NO. He also pointed out that in dry savanna sites of Venezuela high NO fluxes coincided with high soil NO_2^- concentrations (12). Although chemical production of NO from NO_2^- at the low pH values frequently found in savanna sites is likely, an additional source of NO at these sites might be biological reduction of nitrite during nitrification.

The heterotrophic nitrifier A. faecalis ATCC 8750 described in this paper behaved somewhat differently than the (A. faecalis strain) characterized by Papen et al. (20) (strain DSM 30030). In our study NO_3^- was not produced during nitrification, and although NO_2^- was found in the medium,

FIG. 10. Growth and production of NO and N_2O by A. faecalis under aerobic conditions in the presence or absence of nitrification inhibitors. An early-stationary-phase culture was filtered, washed, and diluted 10-fold with medium containing citrate (4 mM), NH_4^+ (5 mM), and either no inhibitor (O), N-serve (10 μg ml⁻¹ in 0.095% ethanol) (●), 0.095% ethanol alone (▽), allylthiourea (10 μM) (▼), or phenylacetylene (5 μg ml⁻¹ in 0.095% ethanol) (□). Cultures were incubated at 31°C with shaking (125 rpm). O.D. (660 nm), optical density at 660 nm.

its concentration decreased with increasing cell concentration, probably because of declining pO_2 values, whereas Papen et al. observed accumulation of both NO_2^- and NO_3^- to high concentrations. Kuenen and Robertson (15) observed that in chemostat cultures of *Alcaligenes* sp., NO_2^- accumulation was greatest at the highest DO concentration, with little or no accumulation below 20% of air saturation.

Like Papen et al., we observed high concentrations of both NO and N_2O under aerobic conditions, with the ratios of amount of NO produced to amount of N_2O produced varying from 0.06 to 1.1 depending on the pO_2 in the medium and whether the culture was sparged. If NO is a precursor of N_2O, as is generally considered to be the case in heterotrophic denitrification, we would expect the ratio of amount of NO produced to amount of N_2O produced to be higher in sparged cultures and to vary directly as a function of the flow rate of the sparging gas, as was shown to be the case in *Pseudomonas perfectomarina* cultures (31). In unsparged aerobic *A. faecalis* cultures incubated with shaking, we observed a ratio of amount of NO produced to amount of N_2O produced of 0.07 (standard deviation, 0.06; $n = 12$), whereas in sparged, aerobic cultures the ratio of amount of NO produced to amount of N_2O produced varied as a function of pO_2 and ranged from 0.18 to 1.1. In unsparged cultures Papen el al. (20) observed ratios of amount of NO produced to amount of N_2O produced of 0.9 in a rich peptide-meat extract medium and 0.006 in a defined medium. Whereas we observed both production and consumption of nitrite and NO, Papen et al. were not able to detect either nitrite reductase activity or nitrate reductase activity in cells from cultures which were actively nitrifying NH_4^+. Given the extreme sensitivity of nitrite and nitrate reductases to even trace levels of O_2, it is undoubtedly very difficult to anaerobically wash and harvest cells in a manner that preserves activity. It is clear from both our results and those of Papen et al. that experiments such as these are best done in a chemostat in which the pO_2 and substrate concentrations can be controlled.

In our study, production of NO by unsparged cultures of *A. faecalis* under anaerobic conditions resulted from the abiotic process of chemodenitrification, although the rates of production were very low compared with the rates observed under aerobic conditions. On the other hand, NO produced under aerobic conditions appeared to be of biological origin. We based this conclusion on the fact that whereas levels of nitrite accumulation in the presence of inhibitors varied greatly, ranging from 3.2 μM with N-serve to 8.6 μM with ethanol to 29 μM with phenylacetylene in ethanol to 32 μM with allylthiourea to 99 μM in the absence of inhibitors, the levels of NO production were similar in all of the flasks. This conclusion needs further testing.

The responses of *N. europaea* and *A. faecalis* to nitrification inhibitors were quite different. In general, *N. europaea* was far more sensitive than *A. faecalis* was. The results of experiments in which nitrification inhibitors were used to identify the process responsible for emission of NO or N_2O must therefore be interpreted with caution. More work must be done to identify inhibitors which distinguish autotrophic nitrification from heterotrophic nitrification.

In those ecosystems in which fluxes of nitrogen trace gases have been measured, there have been only rare attempts to identify the microbial process responsible for the fluxes. In most cases, it is assumed that autotrophic nitrifiers or heterotrophic denitrifiers are responsible for production of NO or N_2O. In forest ecosystems it has been suggested that heterotrophic nitrifiers, primarily fungi, might be re-

sponsible for much of the ammonium oxidized. Bacterial heterotrophic nitrifiers have generally been ignored, yet they are very common in most terrestrial and aquatic ecosystems. Results of Papen et al. (19) suggest that much of the NO and N_2O emitted from forest ecosystems in Germany may be products of heterotrophic nitrification. The results of our comparison of NO and N_2O emissions by autotrophic and heterotrophic nitrifiers described above support the hypothesis that heterotrophic nitrification may be as important a source or more important a source of NO or N_2O in the atmosphere as autotrophic nitrification, at least in some ecosystems. Our ability to develop accurate assessments of total biogenic emissions will depend on our improved understanding of the processes involved.

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